Stoichiometric Measurements of 30S and 50S Ribosomal Proteins from Escherichia coli

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Hans Jürgen Weber

aus Bamberg

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Referent: Prof. Dr. H. G. Wittmann

Korreferent: Prof. Dr. R. Lochmann

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Lebenslauf

12.2.1942	geboren als Sohn von Gerda und Felix Weber in Teplitz-Schönau (CSSR)
1948-1952	Besuch der Volksschule in Bamberg, Bayern
1952-1961	Besuch der Oberrealschule in Bamberg
Juni 1961	Ablegung der Reifeprüfung
1961-1963	Chemiestudium an der Friedrich-Alexander-Universität in Erlangen - Nürnberg
September 1963	Ablegung der Diplomchemikervorprüfung
1963-1967	Fortsetzung des Chemiestudiums an der Technischen Universität Berlin
Juni 1967	Ablegung der Diplomhauptprüfung
1967-1968	Anfertigung einer Diplomarbeit im Fach Lebensmittelchemie
1968-1972	Promotion über ein Thema aus der Molekularbiolo im Max-Planck-Institut für Molekulare Genetik,

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Stoichiometric Measurements of 30S and 50S Ribosomal Proteins from *Escherichia coli**

H. J. Weber

Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, Berlin-Dahlem, Germany

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Summary. Stoichiometric investigations were made for all 21 proteins of the 30S subunit and for 31 proteins of the 50S subunit. While the results for the 30S proteins strongly suggest the existence of two different stoichiometric classes, the so-called "fractional" (F) and "unit" (U) proteins with average molar amounts of 0.1–0.5 and 0.8–1.2 per mole 30S respectively, at least one further group is present in isolated 50S particles. Therefore 50S proteins may be subdivided in the following way: a) 9–10 proteins with average molar amounts of 0.2–0.6 (F-proteins), b) 15–16 proteins with 0.8–1.2 copies per 50S particle (U-proteins), c) at least one protein with an average value of 1.8–2.2 copies, the "repeated" (R) proteins, d) a group of 7 proteins with 1.4–1.7 copies per particle; they can be named "fractional repeated" (FR) proteins. These findings indicate that 50S ribosomal particles show a more differentiated degree of structural heterogeneity at least in vitro than has been shown for 30S subunits.

Introduction

Since the finding of Waller and Harris (1961) that *E. coli* ribosomes have a complicated pattern of perhaps non-identical proteins, much progress has been done in fractionating them (Kaltschmidt *et al.*, 1967; Traut *et al.*, 1967; Fogel and Sypherd, 1968; Moore *et al.*, 1968; Hardy *et al.*, 1969; Traut *et al.*, 1969; Hindennach *et al.*, 1971a, b; Mora *et al.*, 1971). Careful chemical and physical characterizations of isolated components indicated that 21 individual proteins can be isolated from 30S and 34 different proteins from 50S ribosomal subunits (Craven *et al.*, 1969; Dzionara *et al.*, 1970; Kaltschmidt *et al.*, 1970; Kaltschmidt and Wittmann, 1970b; Mora *et al.*, 1971).

As the molecular weights of all 30S proteins became known, it turned out that the total sum of the molecular weights of the 21 isolated proteins ($\sim$400000 Daltons) was inconsistent with the conclusion that each protein molecule occurs in each 30S ribosomal subunit. One mole of 30S particles only contains about 260000 Daltons of protein (Craven *et al.*, 1969). Measurements of the average number of copies for each protein per 30S particle established the existence of at least two different stoichiometric classes. One group of proteins is present in amounts of 1 mole per mole 30S subunit, another group in much smaller amounts, varying from 0.1 to 0.6 copies per 30S particle (Voynow and Kurland, 1971). These results suggested the existence of structural heterogeneity in purified 30S ribosomal populations.

* Paper No. 35 on "Ribosomal Proteins". Preceding paper is by E. Deusser and H. G. Wittmann, Nature **238**, 269 (1972).

After this finding two further problems became of special interest: 1) Whether the population of 50S particles show similar structural heterogeneity as the 30S subunits, although the protein mass of one particle is large enough to fit in one molecule of each 50S ribosomal protein (Dzionara *et al.*, 1970; Mora *et al.*, 1971). 2) Whether such heterogeneous populations exist not only *in vitro* but also *in vivo* and what their biological meaning could be.

This paper is mainly concerned with the stoichiometry of 50S ribosomal proteins. It is possible to separate simultaneously all 30S- and 50S-, as well as almost all 70S ribosomal proteins in analytical amounts by the two-dimensional polyacrylamide gel electrophoresis technique of Kaltschmidt and Wittmann (1970a). Calculations of the average number of copies per 30S particle reported in this paper are with few exceptions in good agreement with those of Voynow and Kurland (1971). Furthermore, the results for 50S proteins which are presented for the first time, suggest the existence of at least three different stoichiometric classes. Two of them correspond to "fractional" (F) and "unit" (U) proteins found in 30S particles, one further group comprehends the "repeated" (R) proteins with 1.8–2.2 copies per 50S subunit. Seven proteins with 1.4–1.7 copies may be summarized as "fractional repeated" (FR) proteins. From these results structural heterogeneity is also concluded for 50S populations.

Materials and Methods

1. Growth Conditions

Cells of *E. coli B* lys⁻ were grown at 37° C in minimal medium (Apirion, 1966) supplemented by 50 μg/ml of lysine-HCl. In some experiments, *E. coli K* (A 19) cells were grown without lysine-HCl. Labeling was achieved by addition of ^{14}C L-amino acid mixture (N.E.N. 0.25 mC/2.5 ml 0.1 M HCl) to a final activity of 0.04–0.12 μC/ml. Measurements showed that the uptake of radioactivity occurs in 2–3 generations almost completely. Cells were harvested in the middle of log phase by centrifugation and the pellets frozen at −20° C.

2. Isolation of Ribosomal Particles

1 g–2 g cells were disrupted by grinding with the 1.5 fold quantity of alumina powder (alcoa A-305) for 2 min. For the isolation of ribosomal subunits the ground cell paste was suspended in a fourfold quantity of buffer 2 (10^{-1} M K-phosphate, pH 7.8, 10^{-3} M $MgCl_2$, 6×10^{-3} M mercaptoethanol), centrifuged in three low speed steps (2×20 min at $12000 \times g$, 1×20 min at $30000 \times g$) and the pellets discarded. The supernatant (1.0 to 1.5 ml with 150–200 OD_{260} in buffer 2) was applied to a 10–30% linear sucrose gradient in 10^{-3} M Mg-acetate and centrifuged for 15 hours at 19000 rpm in a Spinco SW 27 rotor. Fractions of 0.8 ml were collected and the subunits were pelleted at $160000 \times g$ for 21 hours.

The subunits were recentrifuged on the same gradient for complete separation. In several experiments isolation of 70S ribosomes was done in buffer 1 (10^{-2} M Tris-HCl, pH 7.8, 10^{-2} M Mg-acetate, 6×10^{-2} M KCl, 6×10^{-3} M mercaptoethanol). After three low speed centrifugation steps as described, ribosomes were spun down at $210000 \times g$ for 150 min. The pellets were resuspended in buffer 1 and subjected to a further (low speed + high speed) centrifugation cycle. The 70S particles purified in this way were again resuspended in buffer 1.

3. Fractionation of Ribosomal Proteins and Calculation of their Mass Fractions

a) The ribosomal proteins were extracted by 67% acetic acid in the presence of high Mg^{2+} as described by Hardy *et al.* (1969). An amount of 20 OD_{260} per 30S, 35 OD_{260} per 50S or 60 OD_{260} per 70S particle was taken for each sample and diluted with buffer 1 to a final

concentration lower than 1 %. After stirring at 0° C for 45 min, proteins were separated from insoluble RNA by centrifugation at 26600 × g for 30 min. The extraction was repeated with RNA pellets and the wash fluid was given to the extracted proteins. Volumes of the samples were then reduced to 0.4 ml at 10° C by use of Büchi Rotavapor R, filled in dialysis tubes (from Union Carbide Corp., dry heated to 70–80° C for 2–3 days and rinsed in double distilled water) and dialysed against 4 × 350 ml sample gel for 1.5 hours each.

b) Fractionation of the proteins was performed by the two-dimensional polyacrylamide gel electrophoresis of Kaltschmidt and Wittmann (1970a). The mixture of proteins is first separated in a gel of 8 % acrylamide at pH 8.6 mainly due to differences in molecular charges. In the gel of the second dimension which contains 18 % acrylamide, the proteins are at pH 4.6 separated mainly according to size differences (see Fig. 1). After staining of the fractionated proteins in the 2-D slabs with amido black (for further details see Kaltschmidt and Wittmann, 1970a) the spots were cut out from the gel and dried at 70° C for two days. To recover the radioactivity nearly completely, dried samples were oxidized by pure oxygen in a sample oxidizer (Packard Instruments model 305) and the $^{14}CO_2$ absorbed in 19 ml scintillation fluid (3 ml ethanolamine distilled twice under low pressure, 9 ml methanol, 10 ml toluene with 0.8 g 2.2 p-phenylenbis-(5-phenyloxazol), 11 g 2.5 diphenyloxazol per liter toluene). After cooling for 20 hours, the samples were counted in a scintillation counter (Packard model 3380), corrected by the use of automatic quench controls and calculated as disintegrations per minute (dpm).

c) The mass fraction for a given individual protein i (mf_i) is calculated by the ratio of detected radioactivity in the separated component i (C_i) to the radioactivity of the unfractionated protein mixture (C_u) applied to electrophoresis. Because C_u nearly equals the sum of radioactivity found in the 2-D slab (C_s) after fractionation, C_s instead of C_u was used throughout these calculations. This provides the advantage of ruling out inaccuracies of pipetting which would create large deviations in C_u. The mass fraction mf_i depends on quantity and size of a uniformly labeled protein. The ratio mf_i to MW_i (MW = molecular weight of component i), however, depends only on the quantity of protein i which had been present in the unfractionated mixture. Therefore, the product of mf_i/MW_i and the total amount of protein per mole of ribosomal particle (P) gives the number of copies N_i.

This is only true, if all of the protein material polymerized in the gel migrates during electrophoresis. However, partial denaturation during preparation, interaction of basic and acidic components in the mixture, inclusion of protein during polymerisation of sample gel may cause variable amounts of protein to remain at the origin of the 2-D slabs. As a consequence "apparent" copy numbers ($N_{i,a}$) should be defined. They vary considerably in different slabs and are in general too low. To get the "real" numbers of copies ($N_{i,r}$) plots can be drawn of ($N_{i,a}$) vs. relative amounts of protein retained at the origin of different slabs (see Fig. 2). The extrapolation to 0% protein retained allowed the calculation of $N_{i,r}$.

4. Control Experiments

a) Purity of Subunits. Separated ^{14}C labeled ribosomal subunits (isolated as described) were rerun on a second gradient of 10–20 % sucrose and the amounts of contamination by either 50S or 30S subunits were estimated planimetrically. As a further control, total amounts of radioactivity applied to 2-D slabs were compared with the sum of radioactivity found in the fractionated proteins of the subunit and in the protein retained at the origin. The possibility that 30S dimers contaminate 50S fractions was checked by co-centrifugation of ^{14}C labeled 30S subunits with unlabeled 50S particles in a mass ratio of 0.5. The planimetric comparison of optical density and radioactivity allowed the calculation of the contamination by dimers.

b) Efficiency of the Acetic Acid Extraction. ^{14}C uniformly labeled cells contain ribosomes labeled in protein and in RNA. It is not possible in such experiments to calculate the amount of protein retained by ribosomal RNA. ^{32}P label, however, cannot be detected in ribosomal proteins (H. J. Rahmsdorf, personal communication) but of course is found in ribosomal RNA. Therefore, cells were used simultaneously labeled with ^{32}P and ^{14}C. During preparation of ribosomal proteins (see above) samples were taken at different steps and the radioactivity estimated. By measuring the total amount of ^{14}C in the RNA pellet (*b*) which is the sum of ^{14}C in RNA (*c*) and in retained ribosomal proteins (*a*), *a* can be calculated by subtraction of *c*

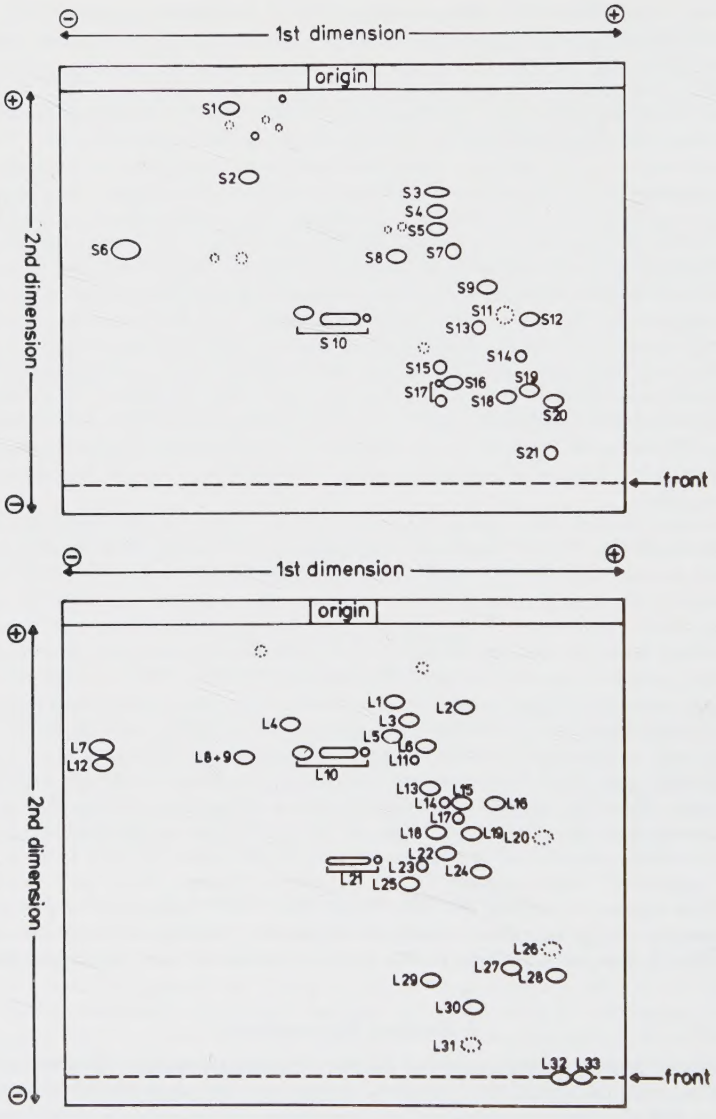

Fig. 1. a and b. Patterns of 30S and 50S ribosomal proteins as obtained by two-dimensional gel electrophoresis. S stands for *small* (30S), L for *large* subunit (50S); numbers for fractionated proteins are taken from Kaltschmidt and Wittmann (1970b). a The mixture of proteins is polymerized in 0.4 ml of sample gel with 2.5–4% acrylamide ("origin"). Proteins are fractionated in 8% acrylamide gel at pH 8.6 (first dimension) and in 18% acrylamide gel at pH 4.6 (second dimension). For further details see Kaltschmidt and Wittmann (1970a). b Protein spots which are marked by dotted circles are only faintly visible.This may be taken as an indication that only small amounts of these proteins are present

from *b*. Because *c* cannot be measured directly it has to be calculated by the following method: The ratio of *c* to ^{32}P is constant in uniformly labeled RNA. Ribosomal RNA is found in the pellet (*d*) as well as in the extracted ribosomal proteins from which it is partly removable by

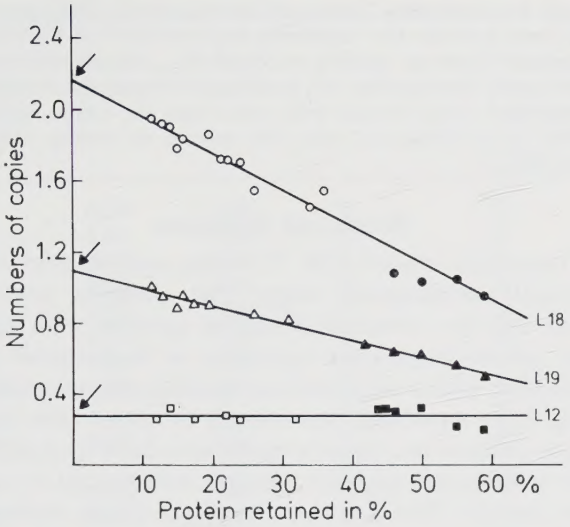

Fig. 2. Calculation of the "real" numbers of copies by graphical extrapolation. Open signs: apparent numbers of copies for 50S particles; full signs: apparent numbers of copies for 70S particles. a There is a linear dependence of the apparent number of copies (ordinate) on the portion of protein retained at the origin of 2-D slabs (abscissa). Extrapolation to 0% protein retained (arrows) gives the "real" number of copies $N_{i,r}$. b Plots were drawn for each investigated ribosomal protein. They show large differences in steepness, indicating that different proteins are retained in different amounts during electrophoresis. This graphical method, which is less complicated than the "isotopic dilution" method used by Voynow and Kurland (1971), allows the correction to 100% recovery (see Material and Methods). c For many proteins copies are the same in 50S (30S) and 70S particles as shown for L18, L19 and L12. Few proteins, however, show differences depending on whether subunits or 70S ribosomes are investigated. Further work on this problem is in progress

dialysis (see Table 4). When e represents the total ^{14}C activity in the sample gel after dialysis of the extracted ribosomal proteins and f the ^{14}C activity corresponding to the small amount of ribosomal protein which might migrate through the dialysis membrane, one can derive

$$\frac{e-f}{g} = \frac{c}{d} \text{ and } c = \frac{d}{g}(e-f)$$

with g for ^{32}P radioactivity in the sample gel after dialysis. Thus, the amount of ribosomal proteins retained by ribosomal RNA after acetic extraction (a) is

$$a = b - \frac{d}{g}(e-f)$$

where b, d, g, and e can be measured directly and f after pressure dialysis through filters Amicon UM2 (see legend to Table 4).

c) Loss of Proteins During Dialysis and Destaining Procedures. Dialysis fluids of different preparation steps as well as destaining baths were filtrated separately through filters which retain molecules larger than 1000 Daltons (Amicon UM-2, pressure filtration). After extensive washing with distilled water, filters were dried at room temperature and burned in pure oxygen as described. Net radioactivity found in filters was compared with total radioactivity of various preparations and relative amounts of loss calculated.

d) Influence of Labeling Conditions. Ribosomal proteins from different cells labeled with either ^{14}C amino acid mixture (aa) or ^{14}C leucine (leu) were subjected to gel electrophoresis

separately. For each fractionation a "degree of heterogeneity" (DH) was calculated. DH is understood as the ratio between the "apparent copy number" of individual protein ($N_{i,a}$) and that of one protein chosen as common standard ($N_{S,a}$). It should be mentioned that in the case of leu-labeling the mass fraction mf_i has to be divided by the number of leu molecules per molecule of protein i. (Data for leu were taken from the amino acid analysis of Kaltschmidt *et al.*, 1970). If no differences exist with respect to labeling conditions, the DH_{aa} should be equal to DH_{leu}.

Results and Discussion

Ribosomes from cells labeled with ^{14}C amino acid mixture were isolated by various differential centrifugation steps. The subunits were separated by sucrose gradients and the extracted ribosomal proteins were subjected to the two-dimensional gel electrophoresis separation of Kaltschmidt and Wittmann (1970a). This method allows simultaneous fractionation of nearly all ribosomal proteins (see Fig. 1) in quantities high enough to detect even small amounts of individual protein components. Measurements were done by scintillation counting after burning of fractionated proteins together with the gel in pure oxygen in a Packard sample oxidizer. This has the advantage of high recovery of radioactivity. Calculation of molar amounts for 50S proteins was performed as described in the Material and Methods section.

For estimation of molar amounts of ribosomal proteins one must know their molecular weights and the protein content P of the ribosomal particle. Careful measurements were made in different laboratories to determine molecular weights (Craven *et al.*, 1969; Traut *et al.* 1969; Dzionara *et al.*, 1970; Mora *et al.*, 1971). The results show relatively good agreement within $\pm 15\,\%$. Together with an error of $\pm 10\,\%$ for the radioactive measurements, one is left with an overall error of 20–25 % (Voynow and Kurland, 1971). This is, however, far beyond the corrections of 200–500 % which would be nessary for example to bring the values of fractional to those of unit proteins (Table 1 and 2).

For P, the molar protein content, the situation is not quite so unambiguous. In this work values of 260000 Daltons for 30S and 550000 Daltons for 50S proteins were taken. P_{30S} was calculated by Hardy *et al.* (1969) and this value was extensively discussed by Voynow and Kurland (1971). P_{50S} with 550000 Daltons is taken from calculations made by Dzionara *et al.* (1970); the value of 520000 Daltons suggested by Mora *et al.* (1971) is not significantly different from 550000 Daltons. As the difference between the two values is only 6% and it is a systematic error for all proteins, it would not influence the concept of heterogeneity.

a) 30S Proteins. In order to test the accuracy of the methods used in this work, stoichiometric measurements for 30S proteins were made and compared with those obtained by other techniques (see Voynow and Kurland, 1971). The results summarized in Table 1 suggest to divide the 30S proteins into the following two classes: a) "Fractional" (F) proteins with copies of 0.1–0.6 per particle. b) "Unit" (U) proteins which are present in molar amounts (0.8–1.2) per mole subunit. Only proteins S3 and S13 are named "marginal" (M) because they have values of 0.65–0.75 and may be either "unit" or "fractional". Considering the different techniques and the experimental errors there is good agreement between these values and those of Voynow and Kurland (1971) for most of the 30S proteins. Discrepancies which may lead to different classification only exist for proteins

Table 1. Stoichiometric data for proteins from 30S subunits of *E. coli B*

Protein	Degree of heterogeneity in %	No of copies	No of copies	Class	Values taken from Voynow and Kurland (1971)	
					Class	Copies
S1	8	0.10	0.10	F	F	0.14
S2	33	0.40	0.35	F	F	0.55
S3	63	0.75	0.70	M	M	0.71
S4	100	1.20	1.20	U	U	0.89
S5	100	1.20	1.00	U	M	0.80
S6	8	0.10	0.10	F	M	0.80
S7	83	1.00	1.05	U	U	0.89
S8	83	1.00	0.90	U	U	0.90
S9	—	—	—	(U)	U	1.06
S10	50	0.60	0.50	F	M	0.79
S11	—	—	—	(F)	F	0.40
S9+11	129	1.55	1.35	(U+F)	—	—
S12	18	0.20	0.20	F	—	—
S13	58	0.70	—	M	—	—
S14	33	0.40	0.40	F	F	0.52
S15	—	—	—	(U)	—	—
S16	—	—	—	(U)	U	0.89
S17	—	—	—	(U)	U	0.83
S15+16+17	288	3.10	2.90	(U+U+U)	—	—
S18	29	0.35	0.30	F	M	0.73
S19	42	0.50	0.43	F	F	0.60
S20	67	0.80	0.80	U	F	0.61
S21	10	0.12	0.11	F	F	0.34

a) Degree of heterogeneity: it is defined as the ratio between $N_{i,r}$ and $N_{s,r}$, where $N_{i,r}$ is the "real" number of copies of protein i and $N_{s,r}$ that of protein S4, chosen as standard. These values are independent of molar protein content P per mole subunit (see Material and Methods).

b) Data in column 3 are calculated with molecular weights from SDS gel electrophoresis measurements (Dzionara *et al.*, 1970) and in columns 4 as well as 7 with molecular weights from equilibrium sedimentation measurements (Hardy *et al.*, 1969).

c) Classification of proteins depends on their number of copies:
U = unit proteins with copies from 0.8 to 1.2,
F = fractional proteins with copies from 0.1 to 0.6,
M = marginal proteins with copies from 0.65 to 0.75.

d) Under the conditions used for 2-D electrophoresis throughout this work, S9 and S11 are not completely separated (Wittmann *et al.*, 1971); the same is true for proteins S15, S16 and S17 (see Fig. 1). Therefore, copies are calculated as sums S (9+11) and S (15+16+17) with 1.35 and 3.1 respectively. Comparison with the values determined by Voynow and Kurland (1971) and by using 1.0–1.2 copies for S15 as found in few experiments with fairly good separation, proteins S15, S16 and S17 are suggested to belong to the U-class. Similar considerations were made for S9 and S11. The classifications for S9, S11 and S15, S16, S17 are written in parenthesis.

S6, S10, S18 and S21. For protein S6 no direct measurement was made by Voynow and Kurland (1971). This might be one reason for the different value reported in their paper. Furthermore, it should be considered that the estimation of the number of copies, especially for S6 and S21, depends on growth conditions

Table 2. Stoichiometric data for proteins from 50S subunits of *E. coli B*

Protein	Degree of heterogeneity in %	Copies	Class	Protein	Degree of heterogeneity in %	Copies	Class
L1	50	1.10	U	L19	48	1.05	U
L2	41	0.90	U	L20	—	—	(F)
L3	64	1.40	FR	L21	39	0.85	U
L4	41	0.90	U	L22	76	1.65, 2.00[2]	FR, R[2]
L5	66	1.43, 1.80[1]	FR, R[1]	L23	55	1.20	U
L6	53	1.15	U	L24	73	1.60	FR
L7	73	1.70[3]	FR[3]	L25	64	1.40	FR
L8+9	64	1.40	(F+F) or (F+U)	L26	9	0.20	F
				L27	34	0.75	M
L10	44	0.95	U	L28	21	0.45	F
L11	53	1.15	U	L29	50	1.10	U
L12	15	0.30[3]	F[3]	L30	41	0.90	U
L13	64	1.40	FR	L31	—	—	(F)
L14	—	0.80	U	L32	(28)	(0.60)	F
L15	—	—	(U)	L33	(23)	(0.50)	F
L16	37	0.80	U	L34	—	—	(F)
L17	55	1.20	U	L7+12	96	2.10[3]	R[3]
L18	100	2.18	R	L14+15	111	2.40	(U+U)

a) Values in column 2 are calculated as described in legend to Table 1. Protein L18 is used as standard.

b) The molecular weights used for calculation of copies are taken from SDS gel electrophoresis (Dzionara *et al.*, 1970).

Exceptions are marked by superscripts:

1. Molecular weight taken from equilibrium sedimentation (Dzionara *et al.*, 1970).
2. Molecular weight taken from equilibrium sedimentation (Mora *et al.*, 1971).
3. For L7 and L12 a value of 12500 (Terhorst *et al.*, 1972) was taken.

c) Proteins L14, L15, as well as L32, L33 were sometimes difficult to separate from each other, proteins L8 + L9 could not be separated under the conditions used in this work. Their values are written in parenthesis.

Proteins L20, L31 and L34 from isolated 50S particles could be only occasionally detected on various 2-D-slabs. Their numbers of copies were not reproducible and therefore omitted. However, they were in no cases found to belong to the U-, FR- or R-classes. Therefore the suggested classification is "fractional" and marked by parenthesis. For L7 and L12 see Results—50S proteins.

(Deusser, 1972) and on the methods for ribosomal preparation (Voynow and Kurland, 1971). This might be another reason for the observed discrepancies because the methods applied in this work differ considerably from those used by Voynow and Kurland (1971). Although values with good reproducibility exist for protein S10 in my experiments, they may be too low. S10 is one of the few proteins which remain in the "soft" sample gel during the first electrophoresis at pH 8.6. This gel, placed between two portions of separation gel (see Fig. 1) contains only 2.5–4% acrylamide and has pores great enough to release protein during dialysis of the first dimension gel between the first and second electrophoretic step. Therefore, protein S10 could be one of the 2–3 proteins which may account for preferential protein loss during dialysis, as can be seen from Table 3

Table 3. Comparison of stoichiometric data for ribosomal proteins isolated from 30S and 50S subunits

Class	30S		50S	
	No	%	No	%
Fractional proteins	10	47	10 (— 9)	30 (—27)
Unit proteins	9	43	15 (—16)	44 (—47)
Fractional repeated proteins	—	—	7	21
Repeated proteins	—	—	1	3
Not to decide	2	9.5	1	3
Sum	21	100	34	100

a) Additional values for fractional and unit proteins of 50S particles are written in parenthesis, because it cannot be decided whether proteins L8 and L9 are F,F or U,F (F,U) respectively.

b) For the comparison of 30S to 50S copies only those values were taken which had been calculated by using molecular weights from SDS-gel electrophoresis measurements (Dzionara *et al.*, 1970).

(see also "control experiments"). Despite the few discrepancies mentioned, the finding of structural heterogeneity in an isolated average 30S population by Voynow and Kurland (1971) is strongly confirmed by my results obtained with quite another technique.

b) 50S Proteins. Stoichiometric measurements for 50S proteins were made for the first time and the data are listed in Table 2. They can be grouped into four classes (see also Table 3): Proteins L12, L20, L26, L28, L31, L32, L33, as well as L8, L9 (or only one of them) correspond to fractional (F) proteins. The group of unit (U) proteins contains L1, L2, L4, L6 (perhaps L8 or) L9, L10, L11, (L14), (L15), L16, L17, L19, L21, L23, L29 and L30. The stoichiometry for eight of these proteins was independently determined by Thammana *et al.* (1972) and found to be in full agreement with the values listed in Table 2.

In addition to the two described classes (F and U) there are two new groups: "Fractional repeated" (FR) with 1.4–1.7 copies are proteins L3, (L5), L7, L13, L22, L24, L25, whereas proteins L18, (L22), (L5) occur as "repeated" (R) structures with 1.8–2.2 copies per particle. Furthermore, proteins L7 and L12 which together gave a sum of at least two copies may also be regarded as repeated structures because they have almost identical primary structures as was shown by both chemical (Kaltschmidt *et al.*, 1970; Terhorst *et al.*, 1972) and immunochemical methods (Stöffler and Wittmann, 1971). In this context, another finding by Kischa *et al.* (1971) affirms the relation between proteins L7 and L12. These two proteins were shown to be involved in G factor dependent GTP hydrolysis *in vitro* which may play a role in the translocation process (Lipmann, 1969). The relevance of the L7/L12 data is discussed in greater detail by Thammana *et al.* (1972).

One might regard the FR-group with some caution. It contains proteins with about 1.4 copies (L3, possibly L5, L13, L25) and with 1.6–1.7 copies (L7, L22, L24). If full use of error analysis is made (see above) then one might argue that

proteins L7, L22 and L24 belong to the R-group which should include proteins with 1.5–2.5 copies per mole particle. However, the total error might be overestimated as the numbers of copies used in this work have been determined from about 20 different 2-D slabs (see Fig. 2). Especially the error for L7 is lower than $\pm 25\%$ because the molecular weight of 12500 Daltons is calculated from peptide analysis (Terhorst *et al.*, 1972) which is the most exact method for determining molecular weights of proteins. Thus it would be too rigid to classify the FR proteins either as "unit" (L3, (L5), L13, L25) or "repeated" (L7, L22, L24). Further work is expected to clarify the existence of the FR-group.

It should be mentioned that all reported values are average numbers. If, for example, in a given population of 50S subunits, protein L25 occurs in average 1.4 copies per particle, possible interpretations can be a) that each particle of the population contains one copy of L25 and further 40% of the particles have a second one, or b) that about 50% of all particles in the population may contain 3 copies of L25 and the other 50% none, or c) various other combinations.

There is at present no possibility to decide between the different forms of distribution, because so far one cannot fractionate different possible subclasses of a ribosomal population and determine the copies for each subclass separately. In this paper the simplest assumption is made, i.e. that for example a number of two copies means two species of one protein component in each ribosomal particle.

The results reported in Table 2 show structural heterogeneity of an isolated 50S population. The data reflect a more differentiated picture than is the case for 30S particles. Not only *E. coli* strain B was used in this work but also strain K (A19). The analysis showed no differences in the numbers of copies between the two strains. Therefore, the finding of structural heterogeneity is not limited to a particular *E. coli* strain.

c) Control Experiments. Careful control experiments are necessary to establish the validity of stoichiometric measurements performed in this work. Occurrence of precursors which are known to have incomplete sets of proteins (Osawa, 1965; Otaka *et al.*, 1967; Sells and Davies, 1970; Homann and Nierhaus, 1971; Nashimoto *et al.*, 1971), contamination of 50S particles by 30S subunits and vice versa as well as specific loss of ribosomal proteins during preparation may create an artificial heterogeneity.

1. Therefore, in the first control experiments, the purity of 30S and 50S subunits was estimated with respect to occurrence of biosynthetic precursors. Cells grown under the same conditions as described (see Material and Methods) were labeled with ^{3}H uridine and a ^{14}C amino acid mixture. The isolated ribosomal fraction was applied to multiple sucrose gradient centrifugation. Analytical quantities of high-labeled ribosomes were used to achieve resolution of particles sedimenting with slightly different velocities than 30 and 50S.

Since ^{14}C amino acids are preferentially involved in protein and ^{3}H uridine in nucleic acid metabolism, one would expect a somewhat different ^{3}H to ^{14}C ratio for incomplete and complete ribosomal particles because of their different protein content. After recentrifugation of 30S and of 50S fractions, 3–5% of well separated particles were determined by planimetric calculations. They might be ribosomal precursors as suggested by their ^{3}H to ^{14}C ratio.

2. Occurrence of 30S particles in the 50S region and vice versa, as well as of 30S dimers in 50S fractions was calculated as described in Material and Methods. From 2–9% of subunits contaminating each other were found by planimetric calculation after the first sucrose gradient centrifugation.

In order to rule out errors generated by contaminations by either precursors or corresponding subunits, most of the experiments described were done with subunits purified by a second sucrose gradient run.

After this rerun only 0–3% contaminations were found. They were calculated by comparison of total radioactivity detected in the 2-D slab to the radioactivity found in protein spots belonging to the class of investigated subunits.

One might argue that these percentages do not represent the true values because of products caused by protein degradation. The second dimension of electrophoresis is performed in a gel of 18% acrylamide in which molecules are preferentially separated according to their size. Thus degraded proteins would migrate faster than undegraded ones and would be detected on 2-D slabs in position different from the ribosomal proteins. Such anomalous spots were not observed to an extent which would influence the results. The conclusion from these experiments is that contaminations or loss of protein by degradation in the range of 0–3% may account for occasional experimental errors but are not sufficiently high to cause the large variations in the determined numbers of copies.

3. Other possible errors might be generated by insufficient purity of fractionated protein components or by specific loss of proteins during extraction from ribosomes or by loss during various dialysis steps. These possibilities were investigated.

Extensive work was done about the purity of ribosomal proteins fractionated by two-dimensional gel electrophoresis (Kaltschmidt and Wittmann, 1970a, b, 1972; Wittmann *et al.*, 1971). From that it can be concluded that all fractionated proteins except those mentioned in legends to Tables 1 and 2 are sufficient pure to calculate stoichiometric data.

As can be seen from Table 4 specific loss of protein during various operations can also be neglected with one exception. Between the first and the second electrophoresis step, the first dimensional gel has to be dialysed against the buffer used in the second electrophoresis. Although it might be assumed that many ribosomal proteins account for this total loss of $\leq 2\%$, the possibility cannot yet be excluded that the loss is caused by only a few proteins. If for example the total loss of $\leq 2\%$ would derive from 5 (or 10) of the 55 ribosomal proteins the determined copy numbers would be too low by 0.2 (or 0.1) copies for unit proteins. Although further investigations are necessary to clarify this point in more detail, the general degree of heterogeneity is not affected by the relatively small modifications of the results which might be necessary after further studies.

4. Finally the influence of labeling conditions was investigated. If ^{14}C amino acid mixture is used as in the experiments reported, differences in pool sizes of various amino acids as well as incorporation of different amino acids into individual proteins may account for different specific radioactivity in fractionated proteins. These differences, however, may simulate different molar amounts and therefore be responsible for artificial heterogeneity. To test for this possibility,

Table 4. Distribution of r-RNA and r-proteins at various stages of protein fractionation

	Amount of r-RNA in %	Amount of r-protein in %
1. Ribosomal subunits after sucrose gradient centrifugation	100	100
2. Extracted protein before dialysis against sample gel	10	~100
3. Extracted protein after dialysis against sample gel	4	(99–) 100
4. Sample gel after dialysis of extracted proteins	6	<1
5. Ribosomal RNA pellet	90	≤0.05
6. Buffer after dialysis of 1st dimensional gels	not estimated	≤2
7. Anode buffer after 2nd electrophoresis	not estimated	<0.3
8. Destaining baths	not estimated	<0.3
9. Amounts retained in origin of 2-D slabs after electrophoresis, staining and destaining	0.5	5–59

a) Values for ribosomal RNA as well as for protein retained after acetic acid extraction are calculated from experiments with $^{32}P/^{14}C$ double label as described in the text.

b) Results for protein fractions in steps 4 and 6–8 are calculated from scintillation measurements after pressure dialysis and oxidation of filters as described in Material and Methods. This is possible, because the comparison between $^{32}P/^{14}C$ double labeling experiments and those performed with ^{14}C label only shows that RNA pieces in the protein fractions are small enough to migrate through filters UM2 (Amicon).

c) Protein values as in step 9 had been calculated for each 2-D slab by the ratio of radioactivity found at the origin to total radioactivity found in the 2-D slab, both measured after electrophoresis, staining and destaining.

results taken from ^{14}C amino acid mixture were compared with those from ^{14}C-leucine label. ^{14}C-leucine was used, because it is one of the few amino acids which are not metabolized into other amino acids during bacterial growth (Roberts *et al.*, 1963). The results of this comparison are shown in Table 5. From 48 analyzed proteins 46 show no significant deviations from expected values, although the leucine content of each protein is only known with an experimental error of $\pm 10\%$. This also supports the conclusion that structural heterogeneity is not an artefact due to the methods used throughout this work.

Although the most critical questions have been discussed one further remark should be made: In this work the assumption is made that all ribosomal proteins have the same specific radioactivity and that the quantity of proteins is related to the numbers of counts. However, one might argue that this is not true and that the finding of heterogeneity is based on the different specific radioactivities of ribosomal proteins. The following points are related to this discussion:

a) Voynow and Kurland (1971) showed that 30S-proteins have the same specific radioactivity within $\pm 5\%$, although their amino acid compositions are different.

Table 5. Comparison of stoichiometric data calculated from experiments performed with ^{14}C-leucine and ^{14}C aminoacid mixture label

Proteins		$\frac{DH_{leu}}{DH_{aa}}$
Number	%	
37	68	0.9–1.1[2]
9	17	0.8–1.2[2]
2 (L28, L29)	~4	0.66[1], 1.45[2]
6 (S11, L6, L20, L26, L31, L34)	11	unknown
Total 54	100	

a) The degree of heterogeneity (*DH*) is defined as follows:

$$DH_{leu} = \left(\frac{N_{i,r}}{N_{s,r}}\right)_{leu} = \left(\frac{dpm_i}{dpm_s}\right)_{leu} \times \frac{\text{No, of leu per mole } s}{\text{No, of leu per mole } i}$$

$$DH_{aa} = \left(\frac{N_{i,r}}{N_{s,r}}\right)_{aa} = \left(\frac{dpm_i}{dpm_s}\right)_{aa} \times \frac{MW_s}{MW_i}$$

leu = experiment performed with ^{14}C leucine label
aa = the same with ^{14}C amino acid mixture label
i = individual fractionated protein
s = one fractionated protein chosen as standard
MW = molecular weight of protein component
dpm = radioactivity found in fractionated proteins

The protein standard S is the same in DH_{leu} and DH_{aa}. When no differences exist with respect to labeling conditions, then $DH_{leu} = DH_{aa}$.

b) Numbers of measurements are given in superscripts. For proteins S11, L6, L20, L26, L31, L34 no amino acid data were available from Kaltschmidt *et al.* (1970) to calculate numbers of leu per protein component.

b) The comparison of labeling conditions (see above) shows that no significant differences were obtained for most of the 30S and 50S proteins by using ^{14}C amino acid mixtures instead of the well defined ^{14}C-leucine label.

c) Results from cell cultures in different media (Deusser, 1972) show that for most of the proteins no differences exist with respect to their quantity, although one uses constant labeling conditions in rich media. This means, that in minimal media with full uptake of radioactivity within three generations no specific dilution occurs by a higher specific turnover of fractional proteins.

d) The comparison of ribosomal proteins from cells grown in different media show that the three proteins S6, S21 and L12 have similar deviations in their ^{14}C/^{3}H ratio (Deusser, 1972), although they have large differences in their amino acid composition.

These arguments exclude the possibility that differences in specific radioactivities of ribosomal proteins occur to a significant degree or that labeling in poor media results in different labeling of proteins which would be responsible for artefacts.

Considering all controls performed during this work, the statement is made that structural heterogeneity exists for 50S as well as for 30S ribosomal particles *in vitro*. For 50S particles this finding may be somewhat surprising because, in contrast to 30S subunits, the protein mass of 50S ribosomes was calculated as high enough to contain one copy of each 50S protein (Dzionara *et al.*, 1970; Mora *et al.*, 1971). One is therefore led to the hypothesis that this unequal distribution of proteins has some functional significance *in vivo*.

d) Conclusions. As discussed above all stoichiometric measurements were done with isolated particles. Usually ribosomes are subjected to many operations until they are pure enough to undergo investigations as described. While isolated ribosomal populations were found to be heterogenous, it is difficult to extrapolate to the *in vivo* situation without the knowledge of what happens from breakage of cells until the last purification step.

Several findings point to the existence of ribosomal heterogeneity also *in vivo* (Kurland *et al.*, 1969; Voynow and Kurland, 1971; van Duin *et al.*, 1972; Deusser, 1972) but unfortunately, no direct proof is available. The isolation of homogenous ribosomal populations which might be correlated to a certain functional state of ribosomes *in vivo*, would help to find out the biological significance of structural heterogeneity. But until now, this approach suffers from experimental difficulties. However, the concept of heterogeneity as found *in vitro* has another interesting aspect which is independent of the existence of heterogeneity *in vivo*: It might be important for functional analysis of ribosomal proteins. During the last three years it became obvious that ribosomal functions depend on highly cooperative interactions between various ribosomal components. Therefore, methods used in functional analysis such as reconstitution of subunits lacking one protein (Nomura *et al.*, 1969), chemical alterations of ribosomes (Craven *et al.*, 1969; Retsema and Conway, 1969) and inhibition of ribosomal functions by protein specific antibodies (Hasenbank *et al.*, 1972) may produce structural changes great enough to obscure the role of individual proteins by overall effects. These difficulties can be overcome by the following method.

It is well known that only a minor fraction of an average ribosomal population functions *in vitro* and one might believe that this has something to do with the occurrence of fractional proteins. Therefore stimulation of specific functions might be achieved by incubating ribosomes with individual fractional proteins. This assay is quite different from those mentioned above because no drastic changes occur in ribosomes. Only the number of working particles should be increased. In this way the specific stimulation may be correlated to certain proteins. Recently, several fractional proteins such as S1 (van Duin and Kurland, 1970), S2, S3, S14 (Randall-Hazelbauer and Kurland, 1972) and S21 (van Duin *et al.*, 1972) were successfully studied by this method.

The concept of heterogeneity which is now found to be valid also for 50S subunits may extend analysis to the unknown role of 50S proteins in ribosomal function.

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Dr. H. J. Weber
Max-Planck-Institut für Molekulare Genetik
D-1000 Berlin 33
Ihnestraße 63
Germany